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(54) Title: CONTROLLING β-AMYLOID RELATED NEURONAL DEGENERATION BY ANTAGONIZING NGF-EFFECTED NEURONAL ACTIVITY

(57) Abstract

Therapeutic methods for antagonizing β-amyloid associated neuronal degeneration as potentiated by NGF or related growth factors. Therapies relying on antagonists of NGF, and antagonists of the interaction of NGF with the NGF receptor, are disclosed.

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ability of candidates to antagonize NGF potentiation of β-amyloid related neurotoxicity.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments of the invention and from the claims.

Description of the Preferred Embodiments

Antagonists useful in this invention are compounds which bind to NGF or the NGF receptor so as to prevent NGF-induced potentiation of β-amyloid associated neurodegeneration. Both NGF and its receptor are well characterized. NGF (mouse)is reported in Scott et al. *Nature* **302**:538-540 (1982) NGF receptor is reported by Radeke et al., *Nature* **325**:593-597 (1987); Chao et al., *Science* **232**:518-521 (1986). NGF and NGF receptor from other mammalian sources can be obtained using standard techniques involving the use of nucleic acid probes from known mammalian NGF or NGF receptor genes, described above to recover cDNA from neuronal mRNA preparations obtained from the desired species.

Functional variants of NGF and NGF receptor which retain the ability to induce neuronal growth and amyloid potentiating effects described above are also included within the meaning of those terms. Those in the field will understand that variants containing conservative modifications of the precise sequences provided by the above-referenced publications will be functional in the invention, and, therefore, use of such modified compounds is within the spirit of this invention.

Alternatively, a mammal (particularly a rodent or a primate) is used to test a candidate compound <u>in vivo</u>. A neurotoxic β-amyloid peptide (e.g. APP or a fragment such as β-(1-40), described below) is introduced into the mammal's central nervous system, (e.g. by cerebral injection or by introduction into cerebrospinal fluid) in

the presence of NGF or other neurotrophic factor. The β-amyloid related neuronal degeneration can be determined by standard neuropathological (e.g. histopathological) techniques.

Antagonists of neurotrophic factors that are sufficiently homologous with NGF to cross-react (compete) for NGF-receptor-induced neuronal proliferation are also included with the invention. For example, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) are homologous to NGF, and antagonists of BDNF and NT-3 are also within the scope of the invention. In particular, BDNF is reported to be functionally cross-reactive with NGF. Rodriguez-Tabor et al., *Neuron* **4**:487-492 (1990). BDNF and NT-3 are reported by Barde et al., *EMBO J.* **1**:549 (1982); Davies, *Trends Neurosci* **11**:243 (1988); Barde, *Neuron* **2**:1525 (1989); Hohn et al., *Nature* **34**:339 (1989); Liebrock, *Nature* **34**:341 (1989); Maisonpierre, *Science* **247**:1446 (1990); Rosenthal, *Neuron* **4**:787 (1990).

Antibodies to nerve growth factors useful in the invention can be obtained by techniques well known to those in the field. For example, a mammal can be challenged with one of the above-described nerve growth factors, and standard techniques can be used for generating hybridomas, and for screening the hybridomas to identify hybridomas producing anti-nerve growth factor antibody. Standard immunopurification techniques can then be applied to hybridoma supernatant to obtain purified anti-NGF antibody.

Other therapeutics useful in the invention are derived from anti-NGF antibodies by standard techniques. For example, the genome of the above-described hybridoma can be probed by known techniques to clone nucleic acid encoding the variable portion of the monoclonal antibody for attachment to a carrier suitable for therapeutic use. See, e.g., Huse et al. *Science*, **246**:12751281 (1989),

hereby incorporated by reference. Other methods of producing NGF-binding molecules from anti-NGF antibodies can also be used. For example, Genex, U. S. Patent 4,946,778 discloses a method for forming single-chain antibodies; Capon et al., *Nature,* 337:529 et seq. (1989) discloses another method for forming antigen-binding molecules based on an antibody to the antigen.

Anti-NGF antibody (or NGF-binding fragments of it) or other antagonist can be formulated in an appropriate buffered saline vehicle and administered to patients at risk for β-amyloid associated neurodegeneration, for example patients exhibiting symptoms that are characteristic of Alzheimer's disease. The antagonist can be administered directly to the central nervous system. For example, the antagonist can be administered using in-dwelling catheters implanted surgically in the ventricals of the head or in a region with access to the cerebrospinal fluid (CSF) of the spinal chord.

It is also possible to administer peripherally antagonists that cross the blood-brain barrier (or that can be adapted to do so). See for example, drug carriers sold by Pharmatec, Inc., Alachua, Florida which can be used to facilitate transport to the central nervous system of peripherally administrated drugs [ADD PATENTS].

Candidates for use in the invention may be screened for their ability to antagonize NGF potentiation of the neurotoxicity of the first 40 amino acids of β-amyloid to cultured neurons (primary rat hippocampal cultures), as described in the following example. The advantages of this culture system are that is enriched in neurons, and neurons can be cultured at low density to facilitate quantification. Neurons can be identified readily morpoholgically and by immunohistochemical markers, as described below.

Culture System

Primary rat embryonic hippocampal cultures were established by a modification of the protocol previously described Banker et al., *Brain Res.* **126**:397-425 (1977); and Banker et al., *J. Comp. Neurol.* **187**:469-494 (1979). The hippocampus was dissected from the brains of embryonic day 18 rat embryos, incubated in 0.5% trypsin (30 min at 37°C), dissociated by gentle trituration, and cultured at a density of 2×10^4 cells per 16-mm polylysine-coated tissue culture well (Costar) in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) iron-supplemented calf serum (Hyclone), 5% (wt/vol) Ham's F-12 nutrient mixture, 2 mM glutamine, 1 mM sodium pyruvate, 2.5% (wt/vol) Hepes, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Cultures were treated with β-(1-40), a polypeptide corresponding the first 40 amino acids of β-amyloid [β-(1-40)] as described in Fig. 3 of Yankner USSN 07/559,173, filed July 27, 1990, and Yankner et al. *Science* **248**:492-495 (1990). The peptide was synthesized on a Milligen peptide synthesizer and purified by reverse-phase HPLC on C_{18} columns; the sequence was confirmed with an Applied Biosystems model 470 sequencer. The β-(1-40) peptide was solubilized and added to the above-described primary rat hippocampal cultures at four days (as described by Yankner et al., *Science*, **248**:492-493 (1990). Scoring was done after 24 hours exposure to β-(1-40).

For scoring and immunohistochemistry, cultures were fixed in 4% paraformaldehyde/0.12 M sucrose for 30 min at 37°C. Pyramidal neurons were readily identified morphologically as cells that elaborated one primary axon and several dendrites during the first 5 days in culture as described Banker et al., *Brain Res.* **126**:397-425 (1977); and Banker et al., *J. Comp. Neurol.* **187**:469-494

(1979). This morphological identification of neurons was confirmed by immunohistochemical staining for neuron-specific markers including the class III β-tubulin isotype, microtubule-associated protein 2, and tau. See, Yankner et al., *Science*, in press; Moody et al., *J. Comp. Neurol.* **279**:567-580 (1989); Caceras et al., *J. Neurosci.* **6**:714-722 (1986); and Binder et al., *J. Cell Biol.* **99**:191 (1984). Identifiable neurons were judged to be viable by intactness of neurites [lack of beading or retraction] and intactness of soma (absence of vacuolar inclusions and exclusion of 0.25% trypan blue). Determinations of viable pyramidal neurons were performed in triplicate 16-mm tissue wells; five 15-mm^2 fields were scored per well. At least 10 neurons per filed or 50 neurons per well were counted in control cultures.

NGF potentiation of β-amyloid associated toxicity is demonstrated as follows. Addition of β-(1-40) at 20 μM resulted in a 40-50% decrease in the number of viable pyramidal neurons relative to untreated control cultures after 24 hours. Treatment with a 1000-fold lower concentration of β-(1-40) -- i.e., 20nM -- had no detectable effect on neuronal viability. Addition of 2.5S NGF (highly purified mouse 2.5S NGF obtained from Boehringer Mannheim) at 10ng/ml together with the higher (20μM) concentration resulted in slightly increased neurotoxicity relative to β-(1-40) alone. When NGF was added together with the lower inactive concentration of β-(1-40) -- (20nM)--, there now appeared a significant neurotoxic response not observed with the low concentrations of β-(1-40) alone. This response was dose dependent over the β-(1-40) concentration range of 10^{-13} to 10^{-12} M.

As further control, various growth factors (acidic and basic FGF, insulin, EGF, PDGF, and IGF-10 had no significant ability to potentiate β-amyloid associated

neurotoxicity at sub-toxic dosages of the latter, even when the growth factors were administered at much higher concentrations than NGF.

To demonstrate the therapeutic effect of NGF and NGF receptor antagonists, a monoclonal antibody against NGF (i.e., a monoclonal antibody against mouse 2.5S NGF clone 27/21, described by Korsching et al., *Proc. Natl. Acad. Sci. USA* **80**:3513-3516 (1983)) obtained from Boehringer Mannheim, was added (final concentration 0.5 μg/ml), see Korsching, et al, Proc Nat'l Acad. Sci. USA 80:3513-3516 (1983), to the cultures with β-(1-40), at the lower non-toxic dose, together with a potentiating dosage of NGF.

The results of the above examples are shown in Figs. 1 and 2.

In Fig.1, the survival of 4-day-old hippocampal neurons with the designated treatments were compared to untreated controls (Bar #1). Survival was determined 24 hours after treatment. As described, NGF potentiates the β amyloid associated toxicity (Bar #'s 2-5), particularly at non-toxic dosages of the latter. As a control, NGF and anti-NGF are not toxic (Bar #'s 7 and 8).

The ability of NGF to potentiate toxicity is concentration dependent as shown in Fig. 2. NGF was active at 0.1-1 ng/ml, with a half-maximal potentiating concentration of 0.2-0.3 ng/ml ($\approx$1 x 10^{-11} M), which is similar to the concentration range reported to be required for the neurite outgrowth response to NGF and for occupancy of the high-affinity NGF receptor. More specifically, Fig. 2 shows NGF concentration dependence for potentiation of β-(1-40) (20 nM), and neuronal survival was determined 24 hr later. Values are normalized to the maximal decrease in neuronal survival (100% toxic response) observed for the combination of NGF

with β-(1-40). Each value represents the mean $\pm$ SEM (n = 10).

The ability of anti-NGF to antagonize the potentiation is shown by Bar 6 of Fig. 1.

As an alternative to the above-described screen using cultured neurons, <u>in vivo</u> animal screening can be used in which the candidates are co-administered with β-amyloid protein or a toxic fragment thereof to create a lesion that can be visualized using cell stain techniques (e.g., immunohistological strains) directed to appropriate markers such as Tau protein or other appropriate cytoskeletal proteins (e.g., using anti MAP-2 antibody or Ala -50 antibody available from Abbot Laboratories in Chicago, IL).

It appears that neurons of the hippocampus and basal forebrain cholinergic nuclei are reported to be among the most severely affected neuronal populations in AD. Without being bound to any theory, one explanation for the vulnerability of NGF-responsive neurons in AD may be a neurotoxic interaction of β-amyloid with NGF. Although β-amyloid alone is reported to exhibit neurotoxic effects in culture at the 10^{-8}-10^{-7} M concentration range, in the presence of physiological levels of NGF, subpicomolar concentrations of β-amyloid become neurotoxic.

In addition, it appears that β-amyloid deposits may cause induction of the NGF receptor in neuronal cell types typically unresponsive to NGF. Such an induction may be difficult to detect if it is transient or results in the degeneration of affected cells. In particular, it appears that neurons degenerated in response to β-(1-40) possess NGF receptors, as demonstrated by immunohistochemical analysis performed with a monoclonal antibody to the rat NGF receptor (clone 192). See, Chandler et al., *J. Biol. Chem.* **259**:6882-6889 (1984); and

Taniuchi et al., *J. Cell Biol.* **101**:1100-1106 (1985). In control untreated hippocampal cultures, only slight immunoreactivity was detected. Neurons treated with NGF alone were not significantly different. However, in cultures treated with β-(1-40), >80% of the neurons showed high levels of immunoreactivity with the NGF receptor monoclonal antibody after 24 hr. NGF receptor induction appeared to be maximal at a concentration of 5×10^{-11} M β-(1-40). When NGF was added together with a low dose of β-(1-40), many of the NGF receptor-positive neurons developed neurodegenerative changes, which included retraction and disruption of neurites and the development of vacuolar inclusions. Some of the neurons exhibiting increased NGF receptor immunoreactivity did not show morphologic evidence of degenerative changes. As a control, exposure of hippocampal neurons to a neurotoxic concentration of glutamate (400 μM), which resulted in widespread neuronal degeneration, was not accompanied by increased NGF receptor immunoreactivity.

Other embodiments are within the following claims.

CLAIMS

1. A method of treating neuronal degeneration associated with pathological accumulation of β-amyloid protein comprising administering an antagonist of NGF-neuronal interaction to neurons of a patient at risk for said neuronal degeneration.

2. The method of claim 1 comprising administering anti-NGF antibody or an NGF-binding fragment thereof to neurons of said patient.

3. The method of claim 2 comprising administering the variable fragment of an anti-NGF IgG class antibody to neurons of said patient.

4. The method of claim 1 comprising administering an NGF receptor antagonist to neurons of said patient.

5. The method of claim 4 comprising administering anti-NGF receptor antibody or an NGF receptor binding fragment thereof to neurons of said patient.

6. A method of screening candidate compounds for the ability to treat β-amyloid related neuronal degeneration by subjecting cultured neurons to NGF in combination with a candidate compound, and determining the ability of the candidate compound to antagonize an NGF-induced effect on the neurons.

7. The method of claim 6 in which said determination is direct.

8. The method of claim 7 in which said determination comprises determining the ability of the

candidate compounds to antagonize NGF potentiation of β-amyloid related neurotoxicity.

9. The method of claim 7 in which said determination comprises determining the ability of the candidate compound to control NGF-related neuronal growth.

10. A method of screening candidate compounds for the ability to treat β-amyloid related neuronal degeneration by subjecting central nervous system neurons of a mammal to NGF in combination with a neurotoxic β-amyloid peptide and a candidate compound, and determining the ability of the candidate compound to antagonize an NGF-induced effect on the mammal's neurons.

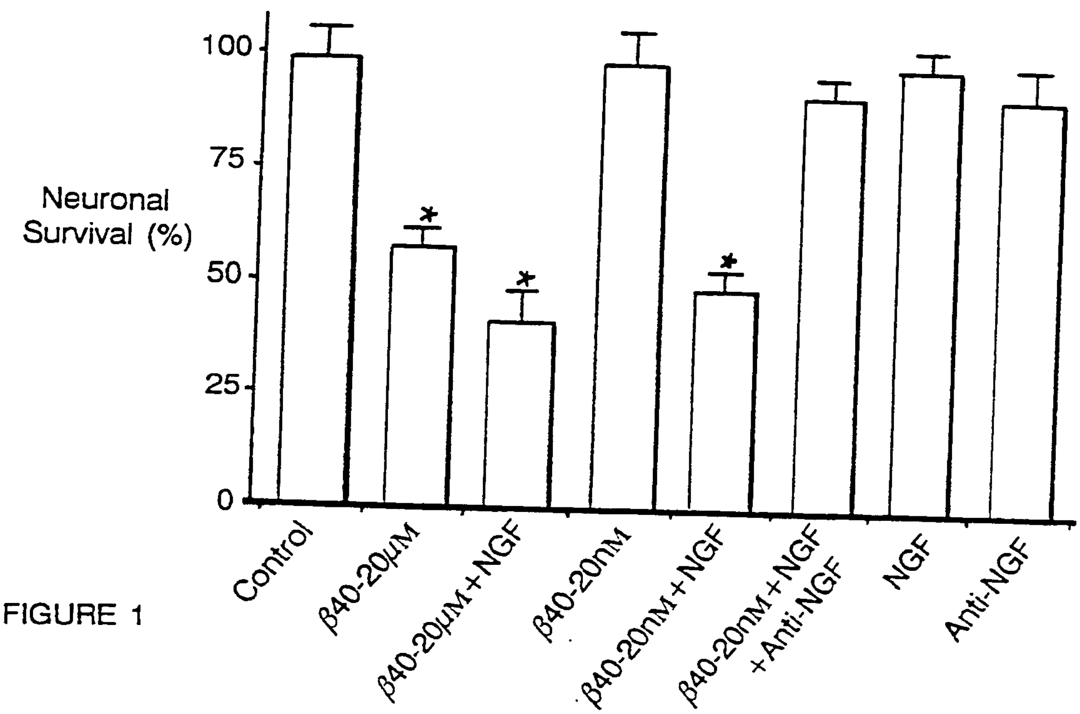

FIGURE 1

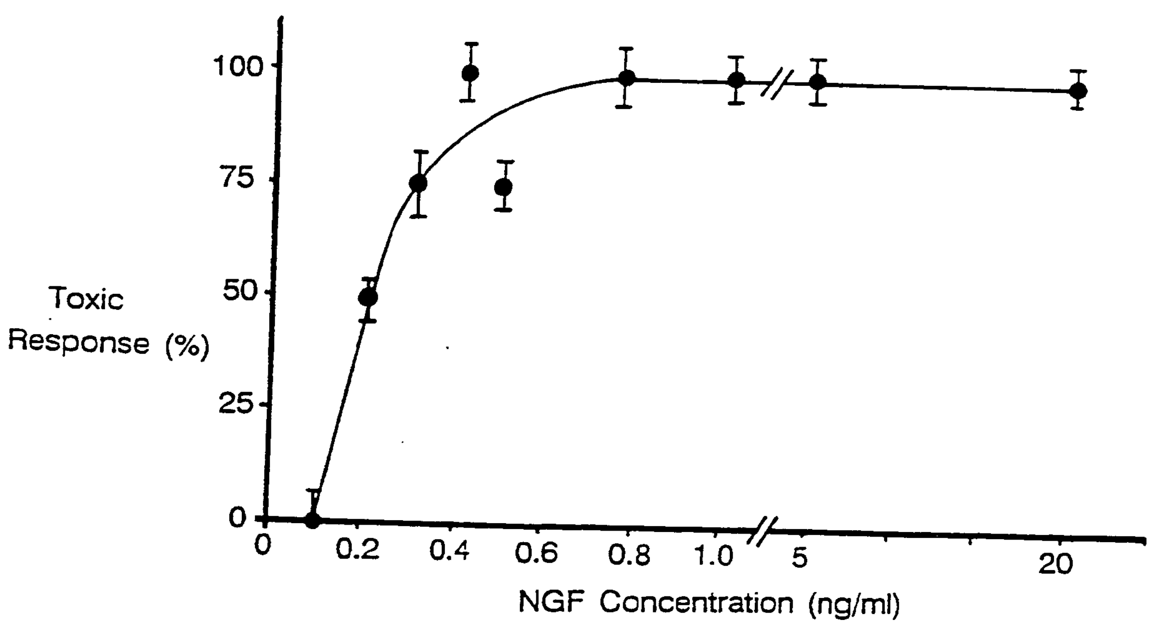

FIGURE 2

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08476

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 39/00
U.S.Cl.: 424/85.8

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/85.8, 570; 530/387, 514/2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AUTOMATED PATENT SYSTEM: DIALOG

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US,A, 4,868,107 (Roy, III et al.), 19 September 1989, see entire document.	1-5
Y	Journal of Biological Chemistry, Volume 259, No. 24, issued 25 December 1984, Bernd et al., "Association of I^{125} Nerve Growth Factor with PC12 Pheochromocytoma Cells", pages 15509-15516. See entire document.	1-5
Y	Proc. Nat'l Acad. Sci., volume 80, issued June 1983, Korsching et al., "Nerve Growth Factor in sympathetic ganglia and corresponding target organs of the rat: Correlation with density of sympathetic innervation", pages 3513-3516. See entire document.	1-5
Y	Science, Volume 243, issued 17 March 1989, Whitson, et al. "Amyloid B Protein Enhances the Survival of Hippocampal Neurons in Vitro", pages 1488-1490. See entire document.	1-5

* Special categories of cited documents: [10]

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29 January 1992	06 MAR 1992
International Searching Authority	**Signature of Authorized Officer**
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Form PCT/ISA/210 (second sheet) (Rev.11-87)

International Application No. PCT/US91/08476

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US,A, 4,701,407, (Appel) 20 October 1987, see entire document.	1-5
Y	US,A, 4,474,892 (Murad et al). 02 October 1984, see entire document.	1-5
Y	US,A, 4,230,691 (Young) 28 October 1980, see entire docuemnt.	1-5